Overexpression of Glutathione Reductase but Not Glutathione Synthetase Leads to Increases in Antioxidant Capacity and Resistance to Photoinhibition in Poplar Trees[1]

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A poplar hybrid, *Populus tremula* × *Populus alba*, was transformed with the bacterial genes for either glutathione reductase (GR) (*gor*) or glutathione synthetase (GS) (*gshII*). When the *gor* gene was targeted to the chloroplasts, leaf GR activities were up to 1000 times greater than in all other lines. In contrast, targeting to the cytosol resulted in 2 to 10 times the GR activity. GR mRNA, protein, and activity levels suggest that bacterial GR is more stable in the chloroplast. When the *gshII* gene was expressed in the cytosol, GS activities were up to 100 times greater than in other lines. Overexpression of GR or GS in the cytosol had no effect on glutathione levels, but chloroplastic-GR expression caused a doubling of leaf glutathione and an increase in reduction state. The high-chloroplastic-GR expressors showed increased resistance to photoinhibition. The herbicide methyl viologen inhibited CO_2 assimilation in all lines, but the increased leaf levels of glutathione and ascorbate in the high-chloroplastic-GR expressors persisted despite this treatment. These results suggest that overexpression of GR in the chloroplast increases the antioxidant capacity of the leaves and that this improves the capacity to withstand oxidative stress.

When plants are subject to stress, whether biotic or abiotic, increased production of potentially dangerous active O_2 species is favored (Asada and Takahashi, 1987; Wise and Naylor, 1987; Polle et al., 1992). To some degree, plants are able to adapt to adverse conditions through altered rates of protein synthesis or repair and through amelioration of their defensive capacity owing to increases in endogenous levels of antioxidative enzymes (Malan et al., 1990; May and Leaver, 1993; Smirnoff, 1993; Edwards et al., 1994; Mittler and Zilinskas, 1994). These responses are generally modest, however, and inadequate in coping with the abundant production of active O_2 species during severe stress conditions, often occasioned by the synergistic action

of high light and an additional stress such as chilling temperature or water deficit (Foyer et al., 1994). Even under moderate (otherwise optimal) light intensities, stress-induced inhibition of CO_2 assimilation will favor increased O_2 reduction in the chloroplast, leading to photoinhibition (Powles, 1984; Gressel and Galun, 1994) and general photooxidative damage (Foyer, 1993).

Many recent attempts to improve stress tolerance in plants have sought to enhance in vivo levels of antioxidative enzymes through genetic transformation (Foyer et al., 1994). Although the efficacy of strategies involving manipulation of single genes remains unproven, several studies have demonstrated greater protection against photo-oxidative stress and increased rates of recovery following photoinhibition in plants overexpressing either SOD or GR (Aono et al., 1993; Sen Gupta et al., 1993a, 1993b; Van Camp et al., 1994). Results obtained for overexpression of SOD are, however, somewhat equivocal: transgenic plants have been produced which, despite having 30- to 50-fold higher SOD activity than untransformed plants, show no improvement in tolerance to the herbicide methyl viologen (Teppermann and Dunsmuir, 1990). Similarly, Pitcher et al. (1991) found no increase in ozone resistance in plants overexpressing SOD. In contrast, expression of a bacterial MnSOD gene in tobacco chloroplasts led to lowered sensitivity to methyl viologen, improved resistance to heavy metals, and increased resistance to drought and low temperatures (Van Assche et al., 1989; Foyer, 1993; Foyer et al., 1994). Furthermore, transgenic tobacco plants overexpressing a gene for SOD from *Nicotiana plumbaginifolia* showed increased tolerance to methyl viologen and ozone (Bowler et al., 1991; Perl et al., 1993; Van Camp et al., 1994), and Sen Gupta et al. (1993a, 1993b) reported that tobacco plants overexpressing chloroplast Cu/ZnSOD were less sensitive to photoinhibition than nontransgenic controls. It is inter-

Abbreviations: AP, ascorbate peroxidase; CaMV, cauliflower mosaic virus; *gor*, coding sequence for GR; GR, GSH reductase; GS, GSH synthetase; *gshII*, coding sequence for GS; *gus*, coding sequence for GUS; MBB, monobromobimane; SOD, superoxide dismutase.

esting that the latter study reported increased endogenous AP activity in plants overexpressing SOD, which may provide an explanation for the conflicting results obtained previously.

SOD converts a relatively harmless derivative of O_2 ($O_2^{\cdot-}$) to one that is much more toxic to the plant (H_2O_2). Hence, increased SOD alone may not render the plant more able to combat oxidative stress. Rather, an increase in H_2O_2-scavenging capacity may also be required to enable removal of H_2O_2 produced by enhanced SOD activity (Foyer et al., 1994). Overexpression of a cytosolic AP increased tolerance to methyl viologen (Pitcher et al., 1994), supporting the conclusion of Sen Gupta et al. (1993b) that increased AP activity was important in determining stress tolerance. Supplementary AP activity during stress conditions might in turn, however, be expected to entail aggravated oxidation of the ascorbate pool. Effective resistance to prolonged stress would then necessitate an augmented capacity for ascorbate regeneration, a process at least partly dependent on the tripeptide thiol GSH in higher plants (Foyer and Halliwell, 1976). Thus, it may well be that sustained enhancement of leaf defenses will ultimately depend on concerted manipulation of antioxidative enzymes. Nevertheless, this purpose is likely to be well served by thorough examination of the defensive significance of individual components, whether SOD, AP, or enzymes of GSH metabolism.

GSH plays several roles in plant defense systems. In addition to its function as an antioxidant, intimately involved with the redox balance of the cell (Kunert and Foyer, 1993), it is instrumental in the detoxification of xenobiotics and heavy metals (Rennenberg, 1982; Alscher, 1989). It is also an important cofactor, both for enzyme activities and for DNA synthesis, as well as being central to the metabolism of reduced sulfur (Schmidt and Kunert, 1986). For this reason, it was of interest to study plants with enhanced capacity for either GSH synthesis or metabolism. Studies of trees are particularly relevant for both ecological and economic reasons. An important crop for timber production, trees have a long life-span and are therefore among the most vulnerable plants, since they suffer from long-term environmental damage. We have transformed poplar trees (*Populus tremula* × *Populus alba*) with the bacterial genes coding either GS or GR to examine the effects of increased activities of these enzymes on GSH and ascorbate metabolism and on the capacity of the leaf to resist oxidative stress. We chose poplar as an experimental

model for study for two reasons: first, because it is a rapidly growing tree of agronomic importance and, second, because an established technology exists for its transformation and micropropagation (Leplé et al., 1992).

MATERIALS AND METHODS

Bacterial Strains

Subcloning of DNA fragments was carried out using the *Escherichia coli* strain JM83recA. The binary vectors were introduced into *Agrobacterium* strain C58pMP90 for transformation (Koncz and Schell, 1986).

Molecular Cloning

The 35 *gor* gene (Fig. 1A) was produced as described by Foyer et al. (1991). For expression of the *gor* gene in the chloroplast, an *Hinc*II/*Eco*RI fragment, excised from the plasmid pKG1 (Foyer et al., 1991), was cloned into the *Sma*I/*Eco*RI sites of the plasmid pJIT117 (Guerineau et al., 1990) to create a translational fusion with the pea *rbc*S sequence coding for a transit peptide. In this vector, the *rbc*S *gor* fusion is flanked by a CaMV 35S promoter sequence containing a double-enhancer sequence and a CaMV poly(A) sequence. The chimeric gene was cloned as a *Kpn*I fragment into the binary vector pBIN19 (Bevan, 1984) to create the vector 70L *gor* (Fig. 1B).

For co-expression of *gor* and *gus* (Jefferson et al., 1987) genes, a *Sac*I fragment containing the *gus* gene (Fig. 1C), the 35S TATA region (−46 to +8), and a 300-bp *Bam*HI/*Bgl*II fragment sufficient for transcription termination were cloned into the unique *Sac*I site of 70L *gor* to create 70L *gor*/*gus* (Fig. 1C). The *gsh*II gene (Fig. 1D) was produced as described by Strohm et al. (1995).

For *gsh*II and *gus* co-expression, a *Sac*I fragment containing the *gus* gene, the 35S TATA region (−46 to +8), and a 300-bp *Bam*HI/*Bgl*II fragment sufficient for transcription termination was cloned into the unique *Sac*I site of 70 *gsh*II to create 70 *gsh*II/*gus* (Fig. 1E).

Plant Transformation

Stem explants of a hybrid poplar clone, *Populus tremula* × *Populus alba* (Institut National de la Recherche Agronomique No. 717-1-B4) were co-cultivated with the disarmed *Agrobacterium* strain C58pMP90. Transformed plants were regenerated on kanamycin-containing media

Figure 1. Representation of the gene constructs used in the poplar transformation. LB, Left border; RB, right border; *nptII*, coding sequence of the neomycin phosphotototransferase gene; L, pea rbcS transit peptide sequence; P35S, CaMV 35S promoter; P70, CaMV 35S promoter with a double-enhancer sequence; P*nos*, promoter of the nopaline synthetase gene; T2, CaMV terminator; T*nos*, terminator of the nopaline synthetase gene; T1, part of CaMV terminator sufficient for termination; P-46 (−46 to +8) CaMV 35S TATA region.
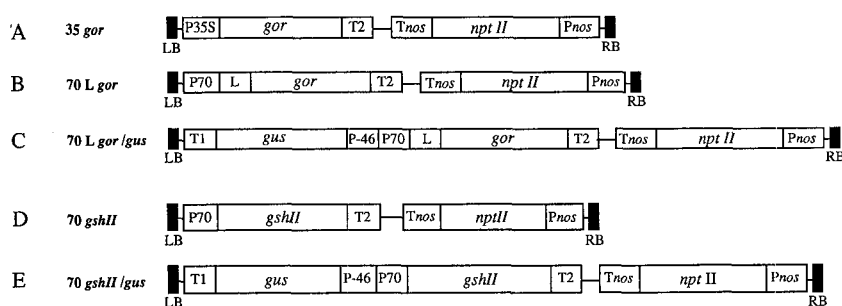
according to the method described by Leplé et al. (1992). Northern analyses were performed according to the method of Tourneur et al. (1993). Lines containing the gene of interest were amplified in vitro before transfer to the greenhouse.

All other molecular characterization procedures were as described by Foyer et al. (1991), Leplé et al. (1992), and Tourneur et al. (1993). The 70L *gor/gus* (Fig. 1C) and 70 *gshII/gus* (Fig. 1E) constructs were prepared to provide an additional marker for selection and also to study the comparative expression of the two genes in poplar. Measurements were made in successive years from 1991 to 1994 on leaves from 1- to 3-year-old trees that were grown in the greenhouse with supplementary lighting (180 μmol m^{-2} s^{-1}) with a 16-h photoperiod.

Nomenclature

In the following discussion, the plants modified to express the *gor* gene in the cytosol are denoted by the prefix 35 followed by *gor* and the number of the transformed line. Those plants expressing the *gor* gene in the chloroplasts have no prefix and are denoted by *gor* followed by the number of the transformed line. Lines expressing both the *gor* and *gus* genes (*gor* in the chloroplast and *gus* in the cytosol) are denoted as *gor/gus* followed by the number of the transformed line. Untransformed controls are denoted as wt. The plants expressing the *gshII* gene in the cytosol are denoted *gsh* followed by the number of the transformed line. Those plants expressing both *gshII* and *gus* genes in the cytosol are denoted *gshII/gus* followed by the number of the transformed line.

Statistical Analysis

All measurements were performed on a minimum of three leaves from between 3 and 10 plants per line depending on the experiment (see figure legends). The results are given either as the mean of the individual values for each plant or as the mean values for each population with the SE = $\sigma_{n-1}/\sqrt{n}$, where σ_n is the SD. The statistical significance of the difference between the mean values was checked by a standard t test. Differences were considered to be significant when the confidence intervals (CIs) for the mean values (CI = $\sigma_{n-1} \times t_{n-1}/\sqrt{n}$, where t is the Student coefficient) calculated for each population with an error of 0.05, showed no overlap.

Measurement of Enzyme Activities and Protein Analysis

Leaves were harvested in the middle of the photoperiod and plunged into liquid N_2. The leaf material was ground in a mortar with liquid N_2, and buffer was added to a volume of about 5 mL/g fresh weight. For GR, the extraction buffer was 0.15 M Hepes (pH 8.0) containing 1.0 mM EDTA and 0.1% Triton X-100. The homogenates were filtered through Miracloth (Calbiochem) and centrifuged at full speed (12,000g) in a bench centrifuge for 5 min. The supernatants were assayed for GR activity by following the decrease in A_{340} upon the addition of GSSG to a final concentration of 1.0 mM in a reaction medium containing 50 mM Hepes buffer (pH 8.0), 1.0 mM EDTA, 0.1 mM NADPH, and extract, in a final volume of 1.0 mL. Protein was determined as described by Bradford (1976).

GS was extracted in buffer containing 0.1 M Tris (pH 7.5), 10 mM MgCl$_2$, and 1 mM EDTA and centrifuged at 16,000g for 5 min, and activity was determined by an adaptation of the method of Rüegsegger and Brunold (1992), in turn adapted from Hell and Bergmann (1988), i.e. HPLC quantification of synthesized GSH as its MBB derivative. After centrifugation, the enzyme activity was assayed in a reaction mixture containing 90 mM Tris-HCl (pH 8.4), 20 mM MgCl$_2$, 45 mM KCl, 4.5 mM DTE, 2 mM Gly, 0.5 mM γ-glutamylcysteine, 5 mM ATP, 5 mM phospho*enol*pyruvate, 5 units of pyruvate kinase, in a final volume of 200 μL. After an incubation of between 10 and 60 min, the reaction was stopped by transferring 25 μL into black tubes containing 40 mM 2-(N-cyclohexylamino)ethane sulfonic acid buffer (pH 8.4) containing 0.6 mM MBB. For each assay, a blank was obtained by taking an aliquot for derivatization prior to incubation. Derivatization was terminated after 15 min by the addition of 1 mL of 5% CH$_3$COOH. Tubes were kept at 4°C and centrifuged at 16,000g. Subsequently, 10 to 50 μL of the derivatized samples were separated on an RP18 column (100 mm long, 4.6 mm i.d., C18 Spheri 5, Applied Biosystems). Compounds were eluted with a linear gradient containing A (10% methanol, 0.25% CH$_3$COOH, pH 3.9) and B (90% methanol, 0.25% CH$_3$COOH, pH 3.9) from 96% A:4% B to 88% A:12% B for 10 min at a flow rate of 1.5 mL/min. MBB derivatives were then detected fluorimetrically (excitation, 380 nm; emission, 480 nm).

Denaturing and nondenaturing PAGE was performed according to the method of Laemmli (1970). Following electrophoresis, gels were stained for GR activity as described by Foyer et al. (1991) or proteins were transferred onto nitrocellulose according to the method of Towbin et al. (1979). Specific antibodies to the bacterial *gor*-derived protein and to the plant forms of GR were used to detect the bacterial and native forms, respectively, as described previously (Foyer et al., 1991).

Gas Exchange Measurements

Net CO_2 assimilation rates in air (340 parts per million of CO_2) were measured on attached leaves in the laboratory using an IRGA (ADC LCA2; Analytica Development Co., Hoddesdon, UK), and the transpiration rates were assessed with a hygrodynamic sensor (Elcowa, 1100, Mulhouse, France). The chamber (ADC PLC, Analytica Development Co.; 6.25 cm^2 surface area, 16 cm^3 volume) incorporated a quantum sensor, together with temperature and humidity sensors. The flexible chamber clip was connected to the IRGA and data acquisition (ADC LCA2) systems. Air was supplied by a mass flow controller (ADC ASU/MF, Analytica Development Co.) with an intermediate mast to prevent perturbations in the gas flow.

Photoinhibitory Treatments

Combined exposure to low temperatures (5°C) and high light (1000 μmol m^{-2} s^{-1}) were used as a standard treat-

ment to induce photoinhibition in poplar leaves. Leaves were introduced into a temperature-controlled chamber with a plexiglass lid, which we have used in previous studies of photoinhibition (Chaumont et al., 1995). Shoots bearing leaves were subjected to treatments of 3 h at 1000 μmol m^{-2} s^{-1} and 5°C. The duration of the treatment varied from 1, 2, 3, and 5 to 15 h, and 3 h was chosen as standard, because, although it caused loss of CO_2 assimilation capacity, the leaves subsequently recovered.

Experimental Procedure

Before and after the application of the photoinhibitory treatment, the light-saturation curve for net CO_2 assimilation was determined. The gas-exchange measurements yielding rates of net CO_2 assimilation were made over a range of light intensities from 10 to 1100 μmol m^{-2} s^{-1}. The slope of the curves obtained at irradiances between 0 and 100 μmol m^{-2} s^{-1} give approximate values of the apparent quantum yield of net CO_2 assimilation.

Ascorbate and GSH Determinations

Leaf discs (10 cm^2) were frozen in liquid N_2 and ground with 1 mL of frozen 2.5 N HClO$_4$. After thawing, the samples were centrifuged for 5 min at 12,000g and the supernatant was neutralized with 5 M K$_2$CO$_3$ to obtain pH values between 4 and 5 and between 6 and 7 for determination of ascorbate and GSH, respectively. Ascorbic acid was measured by the change in A_{265} following the addition of ascorbate oxidase (Foyer et al., 1983). Samples (100–200 μL) of the soluble metabolite fraction from the leaves were added to 0.1 M sodium phosphate buffer (pH 5.6) to make a final volume of 990 μL. The A_{265} was measured in an Aminco (Urbana, IL) double-beam, double-wavelength spectrophotometer and 10 μL (4 units) of ascorbate oxidase (from *Cucurbita*, Boehringer Mannheim) were added. The ascorbic acid content measured via the absorption decrease was estimated using an extinction coefficient of 0.010 at 265 nm. Dehydroascorbate was measured by the same method following reduction to ascorbic acid in a reaction mixture containing 20 mM DTT and 50 mM Hepes-KOH buffer, pH 7.0 (Foyer et al., 1983). GSH and GSSG were assayed according to the method of Griffith (1980). Pheophytin was determined according to the method of Vernon (1960). Chl was estimated according to the method of Arnon (1949).

Methyl Viologen Treatment of Leaf Discs

Leaf discs (10 cm^2) were cut at random from both untransformed and transformed leaves and kept in darkness for 16 h at room temperature in either H_2O or various concentrations of methyl viologen. They were then transferred to the leaf disc O_2 electrode (Hansatech, Kings Lynn, Norfolk, UK). CO_2-dependent O_2 evolution in saturating CO_2 was followed for 20 min. At this point the leaf discs were removed from the electrode and frozen in liquid N_2 for ascorbate and GSH determinations.

RESULTS

Five different constructs (Fig. 1) were used to transform internode fragments of hybrid poplar. For each different construct at least 10 independent transgenic regenerants were obtained. They were micropropagated in vitro, and established cuttings were then transferred to the greenhouse. The initial molecular analysis consisted of the identification of RNA transcripts for *gor* or *gshII* gene expression in the different *gsh*, 35 *gor*, *gor*, and *gor/gus* plant lines (3 or 30 μg of total RNA, Fig. 2, A and B). The *gor* transcripts were about 10 times more abundant in the *gor* plants than in the 35 *gor* plants (Fig. 2A). This difference in the level of expression is probably due to the use of the doubled-enhancer 35S CaMV promoter in the *gor* and *gor/gus* plants and to the unmodified 35S CaMV promoter in the 35 *gor* plants. Leplé et al. (1992) showed that the enhanced 35S CaMV promoter is highly efficient in poplar. The *gshII* (Fig. 2B) and *gshII/gus* (data not shown) plants also showed various mRNA levels (Fig. 2B). Transformed poplar plants with different transcript levels were selected for further molecular characterization and physiological and biochemical analysis.

The gene copy number was estimated by Southern analysis (Fig. 2, C and D). Probes allowing the identification of border fragments (Fig. 2C) and of an internal fragment (Fig. 2D) were used. Some lines contain one copy of the inserted gene, whereas others contain up to five copies (for example *gor* 5). Although the bidirectional nature of the *gor/gus* and *gshII/gus* constructs used for transformation allowed expression of both genes, there was no clear relationship between the relative activities of GUS and GR in the tissues (data not shown).

GR Activity in the Transformed Plants

The foliar GR activities of the plants transformed with the *gor* gene were substantially higher than those of the untransformed controls (Fig. 3). In spite of some variation from year to year, the transformed lines in which the *gor* gene was expressed in the chloroplast (*gor* lines) had more than 100 to 500 times more GR activity than either untransformed controls (Fig. 3A), whereas the transformed plants in which the *gor* gene was expressed in the cytosol under the control of the 35S promoter (35 *gor* lines) had only 2 to 10 times as much GR activity as the controls (Fig. 3B). The foliar GR activities of the plants modified to express the bacterial *gshII* gene (*gsh* lines) were similar to those of the untransformed controls (Fig. 4).

Detection of bacterial GR after SDS-PAGE of soluble leaf extracts by GR activity staining (Fig. 5A) and localization with specific antibodies (Fig. 5B) showed that chloroplastic-GR overexpressors had much higher levels of GR activity and protein than the cytosolic-GR overexpressors. (Fig. 5). Staining the gels for GR activity revealed distinct differences in the position of bacterial GR activity in the transgenic lines expressing the bacterial *gor* gene in the chloroplast and cytosol (Fig. 5A). The bacterial GR was identified by reaction with specific antibodies (Fig. 5B). The soluble protein fraction from the leaves of untransformed
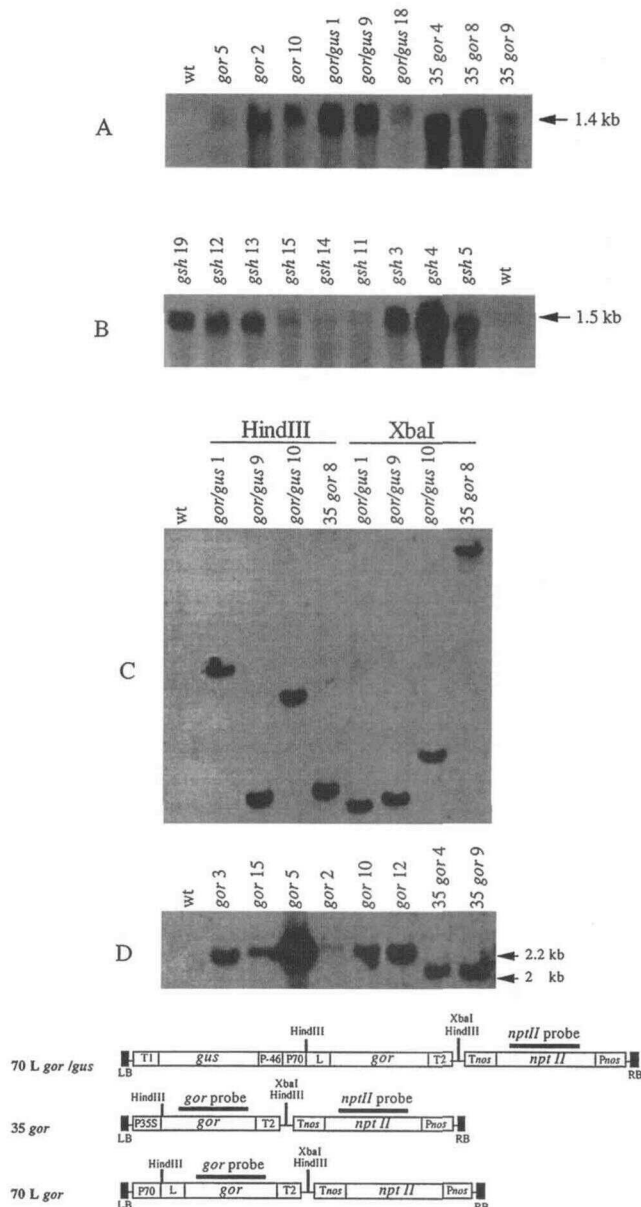
Figure 2. Molecular characterization of transformed poplars. Northern-type hybridization of total leaf RNA from untransformed (WT) and transformed plants (A and B). RNA (30 μg) from plants expressing *gor* or *gshII* in the cytosol (35 *gor* 4, 8, and 9 and *gsh* 3, 4, 5, 11, 12, 13, 14, 15, and 19) or RNA (3 μg) from plants expressing *gor* in the chloroplasts (*gor* 2, 5, and 10 or *gor/gus* 1, 9, and 18) were loaded. The probes used corresponded to the *gor* (A) or the *gshII* (B) coding sequences. Southern-type analyses (C and D) were performed using DNA (10 μg) samples from *gor* or untransformed plants digested with *Hind*III (C and D) or *Xba*I (C). The labeled probes used corresponded to the *nptII*-coding sequence (C) or to the *gor*-coding sequence (D) as shown on the schematic representation of the vectors. The original blots were scanned using Apple Scan on an Apple (Macintosh) computer for visualization. Abbreviations as in Figure 1.
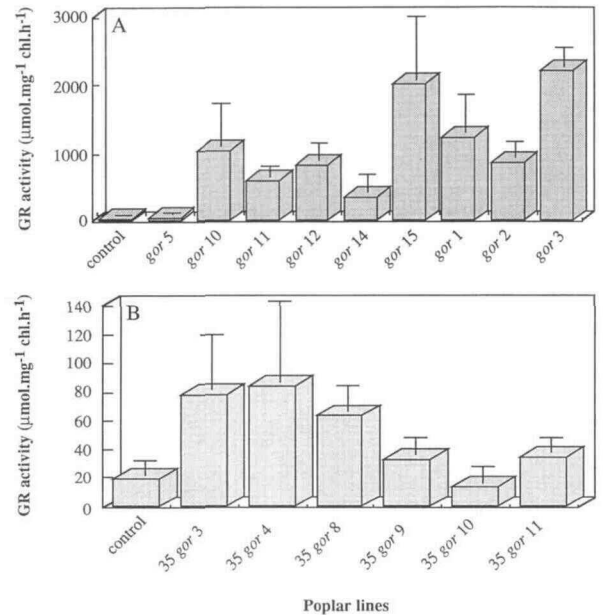

Figure 3. GR activity in the leaves of different lines of transformed poplars expressing the *gor* gene in the chloroplast (A) or the cytosol (B). The foliar GR activities of three plants per transformed line and six plants per untransformed control are given.

plants yielded two closely running bands of native GR activity (Fig. 5A, lanes F and G). When bacterial GR was expressed in the cytosol of different transformed lines (Fig. 5A, lanes A–E), the band of bacterial GR activity ran ahead of the native GR bands (Fig. 5A, lanes A–E). When the bacterial GR was expressed in the chloroplast of different transformed lines (Fig. 5A, lanes H–L), the major band of bacterial GR activity was found between the bands for the native GR and the bacterial GR expressed in the cytoplasm (Fig. 5A, lanes H–L). In addition, the high-chloroplastic-GR expressors (Fig. 5A, lanes H–L) showed a minor band of activity, equivalent in position to the bacterial GR expressed in the cytosol under the control of the 35S promoter. The activity bands coincided with the protein bands detected by specific antibodies (Fig. 5B). The measured expression level of the *gor* gene under the control of the 70
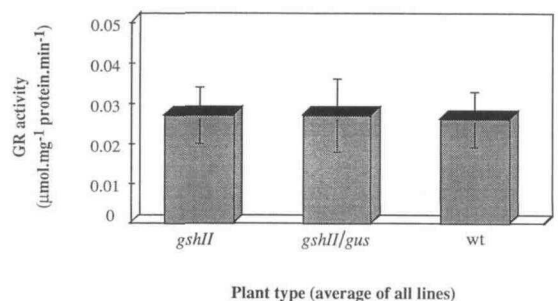

Figure 4. GR activities in all the transformed lines overexpressing GS activity in comparison with untransformed controls (wt). In this study leaf samples from three plants per line were taken from nine lines of *gshII* transformants, seven lines of *gshII/gus* transformants, and six untransformed controls.
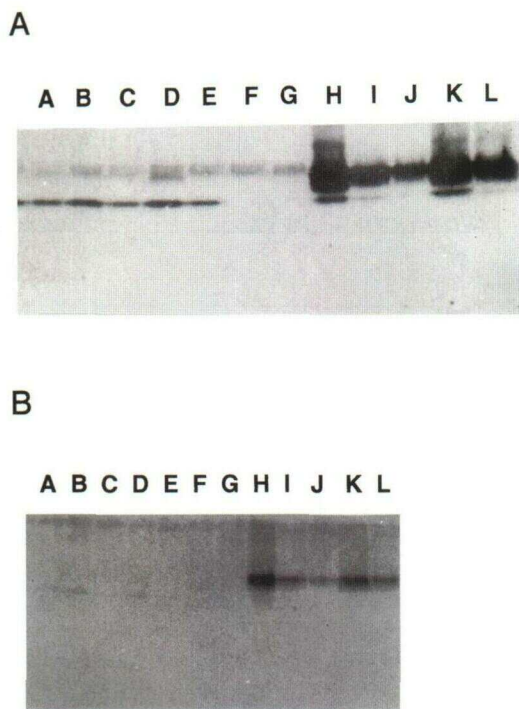
Foyer et al.

A

B

Figure 5. GR activity staining (A) of extracts of total soluble leaf protein on polyacrylamide gels of untransformed poplar (lanes F and G) and transformed lines expressing the *gor* gene in the cytosol (lanes A–E) or in the chloroplasts (lanes H–L). Native and bacterial forms of GR were separated in both cases by nondenaturing PAGE on 15% (w/v) polyacrylamide gels. Extracts of soluble foliar protein (25 µg) were added to each well. Following activity staining, the proteins were transferred to nitrocellulose and probed with specific antibodies to the *gor*-derived protein (B).

promoter was about 10 times that measured for the 35S promoter. The considerable differences in GR activities cannot therefore be explained by differences in the relative levels of expression of each type of construct (Fig. 1). It is interesting to note that the absolute foliar GR activities measured in the leaves in 1994 (see below) were substantially higher than those measured in the sames lines in 1993 (Fig. 3).

GS Activity in Transformed Plants

Foliar GS activity in the lines of plants transformed with the *gshII* gene (*gsh* lines) was considerably higher than that of the untransformed controls (Fig. 6). Lines of plants over-expressing GS activity up to 300 times the level of the untransformed controls were found (Fig. 6). The foliar GR and GS activities were compared in the *gsh* and *gor* lines (Fig. 7). GS activity was constant in the leaves of plants showing enormous increases in GR activities (Fig. 7A). The GR activities were constant in plants that had a wide range of GS activities (Figs. 4 and 7B). There was no evidence to suggest that the activities of these enzymes were coordinated or co-regulated. Like GR activity, foliar GS levels measured in 1994 (Fig. 7) were higher than those measured in 1993 (Strohm et al., 1995).
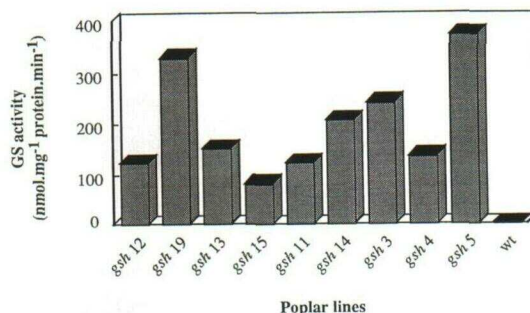

Figure 6. GS activities of individual lines of cytosolic-GS overexpressors. Foliar GS activities in three plants per transformed line were compared to those measured in the leaves of six untransformed (wt) plants. SE values were frequently too small to be visible on the figure and were therefore omitted.

GSH and Ascorbate Pools in the Transformed Lines

To determine GSH and ascorbate contents, leaf samples were harvested between 10 AM and mid-day from plants in the greenhouse. In these experiments the mean GSH content of the leaves of the untransformed controls was 1.0 µmol mg^{-1} Chl, with 96.8% of the GSH present in the reduced form. These values are the lowest point measurements shown on Figure 8. The total foliar GSH pool was increased in the high-chloroplastic-GR expressors compared to the untransformed controls (Fig. 8A). A correlation was observed between the GR activity measured in vitro in foliar leaf extracts and the GSH content of the
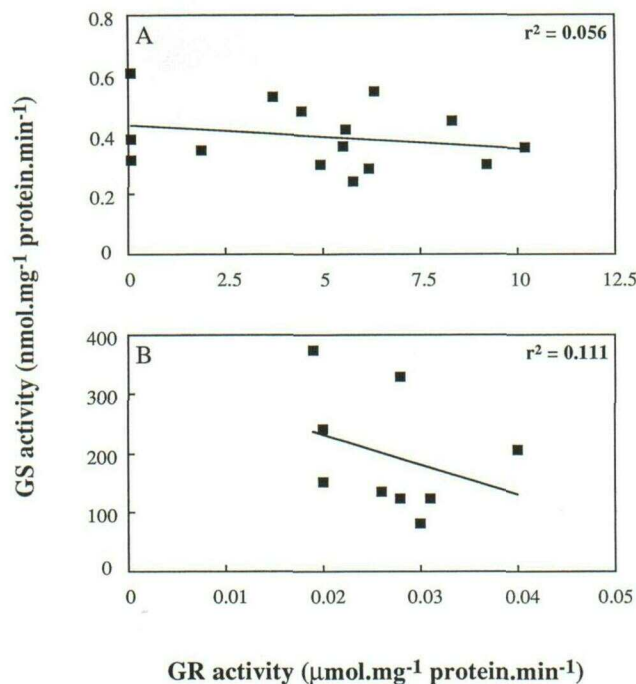

Figure 7. The relationship between foliar GR and GS activities in the leaves of the transformed plants. The total extractable leaf GR and GS activities were measured in 15 lines of plants transformed to express the bacterial GR in the chloroplasts or cytosol (A) or in 9 lines of plants expressing the bacterial GS in the cytosol (B).
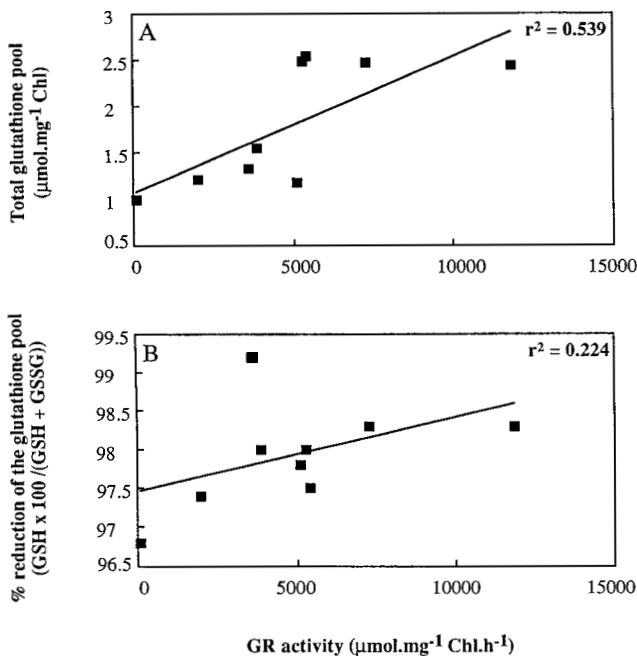
Figure 8. The relationship between foliar GR activity and the GSH content of the leaves. Foliar GR activities and leaf GSH contents were measured in the leaves of eight lines of plants transformed to overexpress GR in the chloroplasts and in the untransformed controls. The total foliar GSH pool (A) and the reduction state of the GSH pool (B) are plotted against GR activity of the leaves.

leaves (Fig. 8A). The level of GSH in the high-chloroplastic-GR overexpressors on a fresh weight or on a Chl basis was always above that of the untransformed leaves and often about twice that of the controls (Fig. 8A). In addition to elevated foliar GSH levels, the high-chloroplastic-GR expressors also showed a small but significant increase in the reduction state of the GSH pool (Fig. 8B).

The high-chloroplastic-GR expressors had higher levels of foliar ascorbate (Fig. 9). Certain lines (Fig. 9A) had more than twice as much ascorbate, on a fresh weight or on a Chl basis, as the untransformed controls or the plants expressing GR or GS in the cytosol (Fig. 9B). The latter all had similar foliar ascorbate levels (Fig. 9B). The reduction state of the ascorbate pool was similar in all types of plants (Fig. 9).

Photosynthesis

The Chl contents of the leaves were similar in all types of plants (Fig. 10A). Photosynthetic rates were similar for all lines of plants when CO_2 assimilation was expressed on a surface area basis (Fig. 10B). Certain lines had less Chl than the other transformed lines and untransformed controls. In these lines, CO_2 assimilation was increased when photosynthesis was expressed on a Chl basis (Fig. 11A) but not on a leaf area basis (Fig. 11B). Overall, however, there were no changes in photosynthesis expressed on a Chl basis when all of the lines of a transformation type were grouped and compared to the untransformed controls (Fig. 10C).

To assess tolerance to oxidative stress, leaf discs from both transformed and untransformed lines were incubated with the herbicide methyl viologen and subsequently illuminated in the leaf disc electrode for 20 min. In the absence of methyl viologen maximum photosynthetic rates were similar in all leaf discs irrespective of the line or plant type (data not shown). In all cases the maximum photosynthetic rate decreased as the methyl viologen concentration increased. Photosynthesis was inhibited to a similar extent in the leaves of all types of plants. The pools of endogenous ascorbate and GSH were relatively insensitive to this brief exposure to saturating light in the presence of low concentrations of methyl viologen. The *gor* and *gor/gus* plants had higher constitutive levels of GSH and ascorbate (Figs. 8 and 9), and this initial advantage persisted following the methyl viologen and light treatments (Fig. 12, B and C). Data are provided only for the two lines whose ascorbate levels are illustrated in Figure 9A, but effects were similar in all cases. These experiments show only short-term effects on CO_2-dependent O_2 evolution and the endogenous reduced ascorbate and GSH levels, but they indicate that the increased antioxidant capacity persists despite the oxidative stress.

Following these observations on oxidative stress tolerance, the response to a more natural stress, photoinhibition, was studied in three lines of each of the untrans-
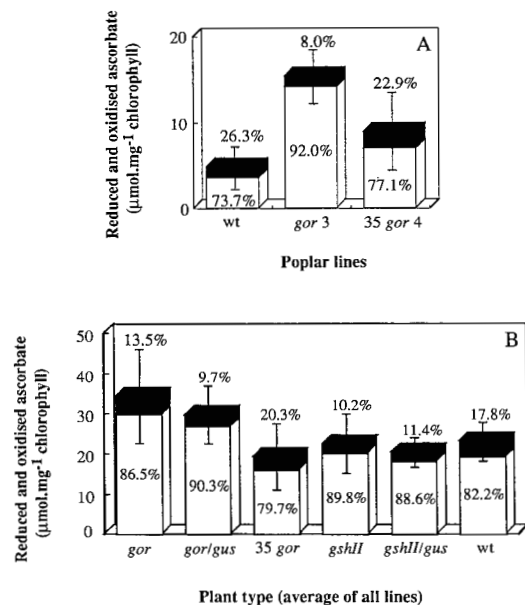

Figure 9. The ascorbate pool of the leaves of transformed and untransformed poplar lines. The clear areas represent the amount of reduced ascorbate present as a percentage of the total ascorbate pool, and the shaded areas represent the amount of oxidized ascorbate. The average ascorbate levels of the highest chloroplastic-GR expressor (*gor* 3) and the highest cytosolic-GR expressor (35 *gor* 4) compared to the values of three untransformed plants were measured in 1993 (A). The foliar ascorbate levels of three plants per line from eight lines of *gor* plants, four lines of *gor/gus*, three lines of 35 *gor*, nine lines of *gshII* plants, and seven lines of *gshII/gus* plants were compared to those of six untransformed controls in 1994 (B).
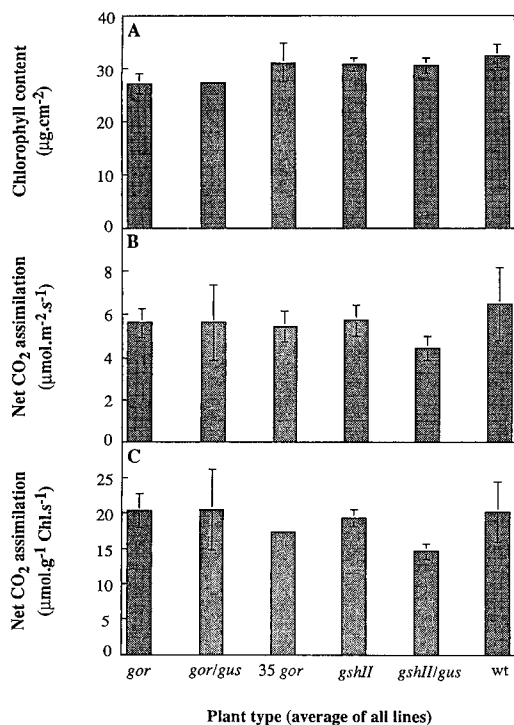
DISCUSSION

In general, the long life cycle of tree species (poplar takes about 4 years to reach sexual maturity) has necessitated the use of only primary transformants for our experiments. There always exists the danger that new traits are related to somaclonal variation consecutive to tissue culture. We have, however, studied large numbers of lines during a 3-year period using samples from small saplings as well as young trees (about 10 feet high). The new characteristics of increased antioxidant capacity and improved tolerance of photoinhibition were found in all lines overexpressing GR in the chloroplasts but not in transformed lines overexpressing bacterial GR or GS in the cytosol. We are, therefore, confident that these characteristics are not due to somaclonal variation but are traits associated with enhanced chloroplastic-GR activity.

Targeting of the bacterial GR to the chloroplastic compartment resulted in the attainment of much greater GR activities than when the bacterial GR was expressed in the cytosol. Activity levels resulting from expression of bacterial GR in the cytosol were consistent with those obtained when the *gor* gene was expressed in *Nicotiana tabacum*
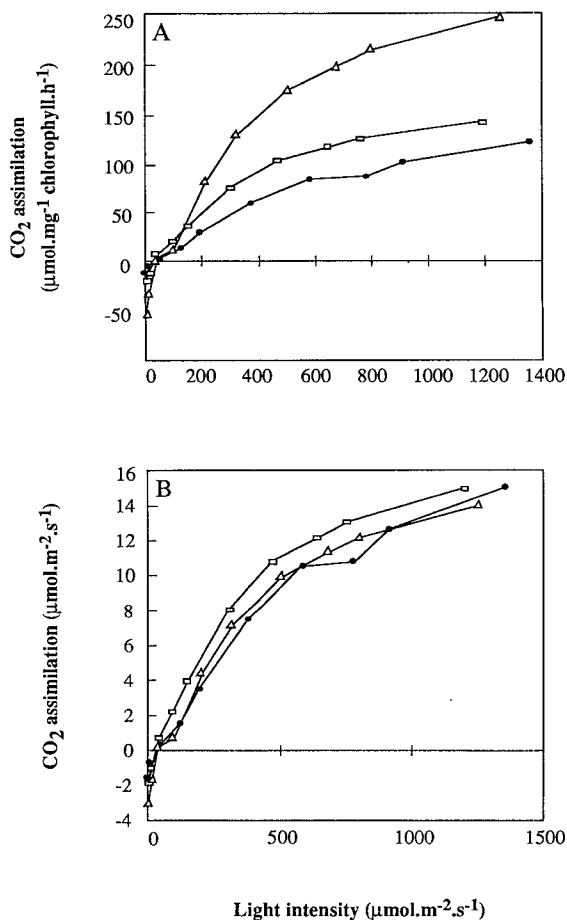

Figure 10. The Chl contents (A), net photosynthetic rates expressed on a leaf area basis (B), and net photosynthetic rates expressed on a Chl basis (C) of all of the lines of poplar plants. The number of samples measured per line and number of lines examined are as for Figure 9.

formed and transformed poplar types. To induce photoinhibition, poplar leaves were subjected to low temperatures (5°C) and high light (1000 μmol m^2 s^1) for 3 h (Fig. 13). There was considerable variation in the susceptibility of individual lines of untransformed controls to this photoinhibitory treatment (Fig. 13). The data of three independent lines of untransformed plants show that despite this natural variation in tolerance a severe inhibition of photosynthetic capacity results from photoinhibitory treatments. In contrast, the response of lines of transformed plants overexpressing GR in the chloroplast was much more uniform. They were much less sensitive to low temperature and high light (Fig. 13A). Before the imposition of the stress all lines showed similar rates of photosynthesis (the upper lines in Fig. 13). Following the photoinhibitory treatment, the slope of the light-response curve for photosynthesis was decreased less and the maximum rate of CO_2 assimilation at saturating light levels was less inhibited in the leaves of the three lines of plants overexpressing GR in the chloroplast (cf. A and B in Fig. 13, lower curves) than in the leaves of untransformed plants under similar conditions. Other lines gave a response similar to the untransformed controls.

Foliar AP activities were constant in all lines, increased GR or GS activity having no measurable effect on total extractable activity (Fig. 14).

Figure 11. Light saturation curves for photosynthesis in untransformed (●), 35 *gor* line 3 (□), and *gor* 1 (△) plants. Rates are expressed as a function of leaf Chl content (A) and leaf surface area (B). Error bars are not given to avoid overlap at the lower light levels.
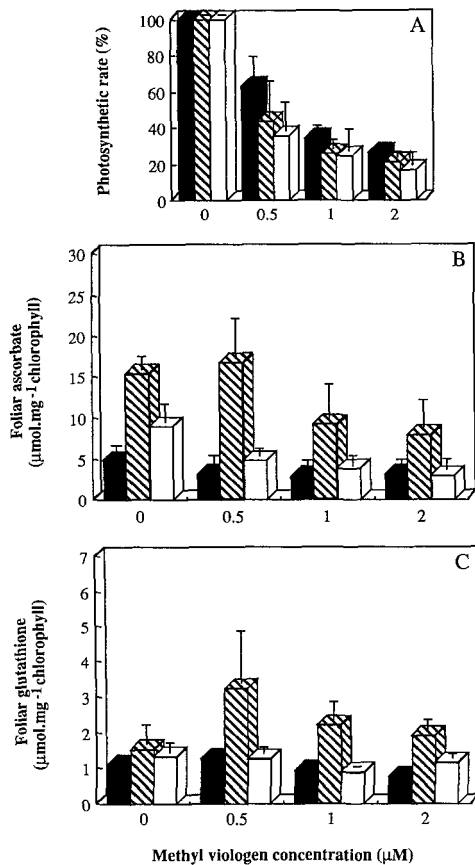
Figure 12. The effect of methyl viologen on photosynthetic O_2 evolution (A) and the reduced ascorbate (B) and reduced GSH (C) contents of leaf discs from untransformed (filled columns), gor 3 (hatched columns), and 35 gor 4 (open columns) poplar plants. Ascorbate and GSH were measured after 30 min of illumination.

under the control of the 35S CaMV promoter (Foyer et al., 1991). Results presented here strongly suggest that GR is more stable in the chloroplast than in the cytosol. This system may provide a means of studying the mechanisms that determine the level of GR activity in two discrete intracellular compartments.

Recent work with N. tabacum (var Samsun) transformed with the GR cDNA from Pisum sativum (Creissen et al., 1991) has shown that overexpression of GR in the chloroplast, but not the cytosol, led to increases in the foliar GSH level by up to 50% (Mullineaux et al., 1994). In the present study, the foliar GSH level was also increased as a result of overexpression of bacterial GR in the chloroplast. Here the total leaf GSH level was about twice that found in the untransformed controls and the cytosolic-GR overexpressors. In contrast, no significant change was observed in foliar GSH content in GS overexpressors, despite increases in GS activities of up to 100-fold relative to control plants. This finding is in accord with previous data (Rennenberg and Polle, 1994; Strohm et al., 1995). We conclude, therefore, that GSH synthesis is tightly controlled in situ. Despite this, elevation of chloroplastic-GR activity can engender considerable increases in foliar GSH and the GSH/
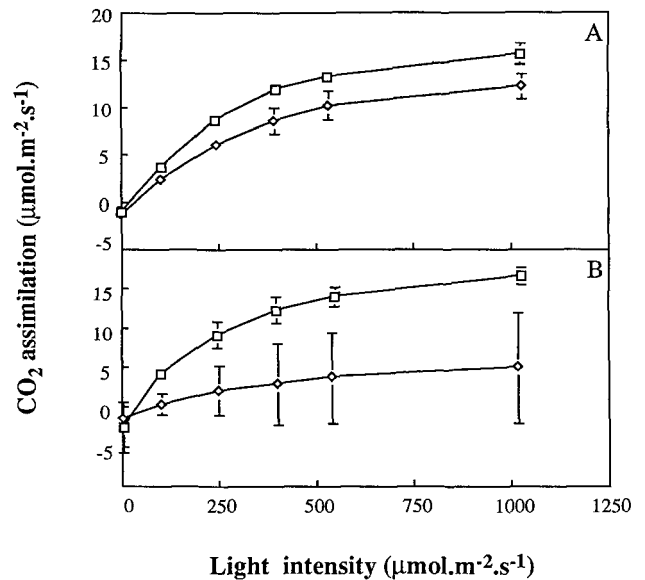

Figure 13. Comparison of the effect of photoinhibitory treatment on attached poplar leaves of different 70L gor lines (A) and of untransformed plants (B). Rates were measured before (□) and after (◇) a 3-h exposure to high light (1000 μmol m^{-2} s^{-1}) and low temperature (5°C).

GSSG ratio, which even in the untransformed controls strongly favors GSH. We are drawn to the conclusion that GSSG is the form of GSH that is marked for degradation and that increased turnover involving regeneration of the reduced form allows higher foliar GSH levels to be maintained.

The increases in foliar GSH apparently led to effects on the foliar ascorbate pool, which was larger, and sometimes more reduced, in the high-chloroplastic-GR expressors. This observation reinforces the view that GSH has an important role in maintaining the ascorbate pool of the chloroplast. Asada (1994) suggested that direct electron transport to monodehydroascorbate, together with NADPH-dependent reduction catalyzed by monodehydroascorbate reductase, is the major route of regeneration of reduced ascorbate in the chloroplast and that GSH-mediated regeneration is less important. The present data support the view that GSH-mediated ascorbate regeneration via the ascorbate-GSH cycle also makes a significant contribution to the
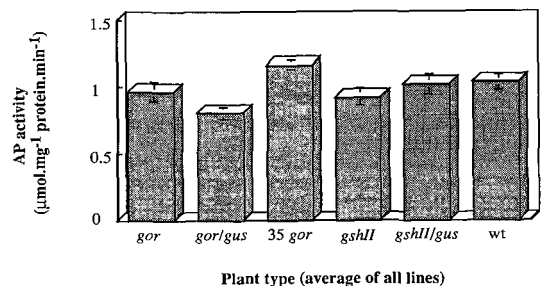

Figure 14. Average AP activities of all lines of transformed and untransformed (wt) plants, measured in the middle of the photoperiod.

size and redox state of the ascorbate pool, as suggested previously (Foyer and Halliwell, 1976; Anderson et al., 1983a, 1983b; Foyer et al., 1991).

Cold-induced photoinhibition occurs naturally in trees even in highly tolerant evergreen species (Bolhar-Nordenkampf and Lechner, 1988) and may hinder the establishment of seedlings and their subsequent development (Ball, 1994). It is thus encouraging to observe that the high-chloroplastic-GR overexpressors had a higher tolerance to cold-induced photoinhibition relative to the untransformed controls. The metabolic basis for this increased tolerance may be multifactorial (Smith, 1985; Schmidt and Kunert, 1986; Smith et al., 1989). First, the increased levels of GSH and ascorbate may confer additional antioxidant protection to the chloroplasts. More rapid recycling of the reduced ascorbate and GSH pools would be beneficial in combating ongoing production of harmful free radicals. Second, increased GSH levels would have implications for all of the cellular thiol-disulfide-exchange reactions (Kunert and Foyer, 1993) and would favor stabilization of enzymes that require reduced thiol groups for activity. Finally, GSH participates in many diverse cellular reactions; augmented levels may exert more indirect effects on protein synthesis and gene expression, which would contribute both to the observed decrease in susceptibility to photoinhibitory treatment and to the enhanced rates of recovery upon removal of the treatment. These results suggest that the high-chloroplastic-GR expressors are more robust as a result of the introduction of bacterial GR into the chloroplast, a conclusion in line with observations on other species transformed to express GR in the chloroplast (Aono et al., 1991, 1993; Mullineaux et al., 1994). This may prove to be of major ecological and practical significance and may have implications for improving methods of tree establishment. The improved stress resistance observed in the *gor* lines could, in the future, constitute part of a tree improvement program. The results presented here suggest that enhanced chloroplastic-GR activity, for example, may be stably incorporated for release of transformed trees to the field.

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